Remarks

Claims 1-42 are canceled. New claims 43-54 are added. Support for the new claims may be found throughout the application as originally filed.[1] These claims are also supported, for example, in U.S. Application No. 09/585,077, now U.S. Patent No. 6,743,823 ("'823 patent"), which is the parent of the instant application.[2] All of the new claims read on the elected species of pulmonary hypertension.

Claims Rejections - 35 U.S.C. § 112, First Paragraph (Written Description)

Claims 1, 2, 4, 5, 8-10, 19, 21-23, 26, 28, 29, 35, 38-40, and 42 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. In particular, the USPTO contends that the specification does not provide a basis for various recitations of "decreased plasma citrulline."[3]

Without acquiescing to the correctness of the rejection, Applicants have canceled claims 1, 2, 4, 5, 8-10, 19, 21-23, 26, 28, 29, 35, 38-40, and 42. Accordingly, this rejection is moot.

Applicants note that new claims 43-54 do not recite "decreased plasma citrulline." Also, in view of the support cited herein, Applicants respectfully submit that the specification provides adequate written description of the full scope of the new claims.

Claims Rejections - 35 U.S.C. § 112, First Paragraph (Enablement)

Claims 1, 2, 4, 5, 8-10, 19, 21-23, 26, 28, 29, 35, 38-40, and 42 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement.

Without acquiescing to the correctness of the rejection, Applicants have canceled claims 1, 2, 4, 5, 8-10, 19, 21-23, 26, 28, 29, 35, 38-40, and 42. Accordingly, this rejection is moot.

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[1] *See, e.g.*, Specification, page 46, lines 9-11 and 20; page 46, line 23 to page 49, line 4; page 50, line 9 to page 51, line 32; Examples 7 and 8; original claims 1, 2, and 4-10.

[2] *See* '823 patent, col. 28, lines 9-13, 22, and 25-45; col. 29, lines 19-61; col. 30, line 14; Example 7.

[3] *See* Office Action, pages 6 and 7.

Applicants also provide the following comments.

The specification teaches that certain disorders such as pulmonary hypertension (e.g., persistent pulmonary hypertension ("PPHN")) are associated with sub-optimal urea cycle function such as that associated with decreased citrulline and/or arginine production.[4] Indeed, for example, the USPTO acknowledges that the specification teaches that "infants who developed PPHN had significantly lower serum arginine and citrulline levels."[5] The specification also teaches that the administration of citrulline "significantly" increases levels of arginine, a nitric oxide intermediate.[6] As such, one of skill in the art, reading the specification, would reasonably conclude that that the administration of citrulline may be used to treat or prevent disorders associated with sub-optimal urea cycle function such as pulmonary hypertension and PPHN.

The specification also teaches that the exposure to certain environmental stimuli such as cardiac surgery (e.g., repair of congenital heart defects) are associated with sub-optimal urea cycle function.[7] Cardiac surgery in turn may be associated with postoperative complications such as increased postoperative pulmonary vascular tone.[8] Accordingly, one of skill in the art, reading the specification, would reasonably conclude that that the administration of citrulline may be used to treat or prevent disorders associated cardiac surgery (e.g., repair of congenital heart defects, cardiac surgery associated with increased postoperative pulmonary vascular tone).

The specification's teachings have been confirmed in post-filing date, peer-reviewed articles. For example, Smith et al.,[9] teaches that (1) the administration of oral citrulline "increased postoperative plasma citrulline and arginine concentrations"; and (2) "increased plasma citrulline concentrations were associated with a decreased risk of

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[4] *See, e.g.*, Specification, page 46, lines 9-11, 13-14 and 20; Example 7.

[5] *See* Office Action, pages 6 and 7.

[6] *See* Specification, Example 8.

[7] *See id.* at page 46, lines 23-28 and 31-32.

[8] *See id.* at page 46, lines 28-29.

[9] Smith *et al.*, J Thor and Cardiovasc. Surg. 2006; 132(1):58-65, attached herewith as **Exhibit A**.

postoperative pulmonary hypertension."[10] Smith et al. also concluded that postoperative pulmonary hypertension did not occur in children with elevated citrulline levels "through supplementation."[11]

Barr et al.[12] teaches that the administration of intravenous citrulline provided similar results. Specifically, Barr et al. teaches that patients undergoing cardiac surgery and receiving intravenous citrulline not only tolerated the citrulline, but also maintained pre-operative levels of citrulline, arginine, and nitric oxide.[13]

Ananthakrishnan et al.[14] teaches that citrulline ameliorates chronic hypoxia-induced pulmonary hypertension in newborn piglets. Ananthakrishnan et al. generated a model of pulmonary hypertension by inducing hypoxia in piglets—"an excellent species for the study of neonatal pulmonary hypertension."[15] Piglets receiving oral citrulline demonstrated lower pulmonary arterial pressures and pulmonary tensions when compared to control piglets.[16] Ananthakrishnan et al. concludes that citrulline supplementation "may benefit neonates exposed to prolonged periods of hypoxia from cardiac or pulmonary causes."[17]

In view of the specification and the post-filing date evidence provided herein, Applicants respectfully submit that the full scope of the claimed invention is enabled.

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[10] *Id.* at page 62.

[11] *Id.* at page 58.

[12] *See*, Barr *et al.*, J Thor and Cardiovasc Surg. 2006; 134(2):319-326, attached herewith as **Exhibit B**.

[13] *See id.* at page 319.

[14] *See*, Ananthakrishnan et al., Am J. Physiol Lung Cell Mol Physiol. 2009; 297:L506-L511, attached herewith as **Exhibit C**.

[15] *Id.* at page L506.

[16] *Id.* at Abstract.

[17] *Id.* at page L506.

CONCLUSION

Should there be any minor issues outstanding in this matter, the Examiner is respectfully requested to telephone the undersigned attorney. Early passage of the subject application to issue is earnestly solicited.

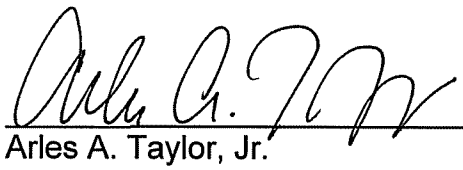
DEPOSIT ACCOUNT

The Commissioner is hereby authorized to charge any fees associated with the filing of this correspondence to Deposit Account Number **50-0426**.

Respectfully submitted,

JENKINS, WILSON, TAYLOR & HUNT, P.A.

Date: 02/28/2011

By: 

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Nitric oxide precursors and congenital heart surgery: A randomized controlled trial of oral citrulline

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CHD

Dr Smith

Objective: The study sought to determine whether citrulline supplementation, a precursor to nitric oxide synthesis, is safe and efficacious in increasing plasma citrulline concentrations and decreasing the risk of postoperative pulmonary hypertension.

Study Design: Forty children, undergoing cardiopulmonary bypass and at risk for pulmonary hypertension, were randomized to receive 5 perioperative doses (1.9 g/m^2 per dose) of either oral citrulline or placebo. Plasma citrulline and arginine concentrations were measured at 5 time points. Measurements of systemic blood pressure and presence of pulmonary hypertension were collected.

Results: Median citrulline concentrations were significantly higher in the citrulline group versus the placebo group immediately postoperatively (36 μmol/L vs 26 μmol/L, $P = .012$) and at 12 hours postoperatively (37 μmol/L vs 20 μmol/L, $P = .015$). Mean plasma arginine concentrations were significantly higher in the citrulline group versus the placebo group by 12 hours postoperatively (36 μmol/L vs 23 μmol/L, $P = .037$). Mean systemic blood pressure did not differ between groups ($P = .53$). Postoperative pulmonary hypertension developed in 9 patients, 6 of 20 (30%) in the placebo group and 3 of 20 (15%) in the citrulline group ($P = .451$), all of whom had plasma citrulline concentrations less than age-specific norms. Postoperative pulmonary hypertension did not develop in patients who demonstrated plasma citrulline concentrations in excess of 37 μmol/L ($P = .036$).

Conclusions: Oral citrulline supplementation safely increased plasma citrulline and arginine concentrations compared with placebo after cardiopulmonary bypass. Postoperative pulmonary hypertension did not occur in children with naturally elevated citrulline levels or elevations through supplementation. Oral citrulline supplementation may be effective in reducing postoperative pulmonary hypertension.

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Pulmonary hypertension is a potentially severe complication after congenital heart surgery that can lead to right-ventricular failure, reduced cardiac output, and death.[1] Treatment options include oxygen administration, induced alkalosis, sedation, paralysis, inotropic support, and parenteral or inhaled vasodilators.[2]

Nitric oxide (NO) causes cyclic guanosine monophosphate-mediated vasodilation of the pulmonary vasculature. Endogenous NO is produced from the metabolism of citrulline and L-arginine, amino acids generated by the urea cycle (Figure 1).[3] Cardiopulmonary bypass leads to significant reductions in postoperative concentrations of citrulline and arginine,[4] and dysfunction of the pulmonary endothelium.[5,6]

Intravenous L-arginine has been shown to reduce both pulmonary and systemic pressures.[5,7] This global response would not be tolerated in patients after cardiac surgery, who are already prone to low cardiac output states. The maintenance of high plasma arginine concentrations is also problematic because of poor bioavail-

Abbreviations and Acronyms
IQR = interquartile range
NO = nitric oxide
PCCU = pediatric critical care unit

ability and swift metabolism by intestinal and cytosolic arginase.[8] In contrast, administration of oral citrulline is more effective in maintaining plasma L-arginine concentrations than administration of arginine in healthy volunteers.[9] Citrulline has no recognized toxicity and is used as replacement therapy for children with urea cycle defects.

The purpose of this study is to determine whether perioperative oral citrulline supplementation is (1) safe in patients after heart surgery, (2) efficacious in increasing plasma citrulline and arginine concentrations, and (3) associated with development of postoperative pulmonary hypertension.

Methods

Patient Enrollment

Vanderbilt Institutional Review Board approval was obtained before patient enrollment. Forty patients were prospectively enrolled in this randomized, placebo-controlled, doubled-blinded study at Vanderbilt Children's Hospital between April 2003 and September 2004.

All infants or children less than 6 years of age undergoing 1 of 6 surgical procedures for correction of congenital heart lesions were eligible for enrollment. Procedures included (1) ventricular septal defect repair, (2) atrioventricular septal defect repair, (3) bidirectional Glenn procedure, (4) modified Fontan procedure, (5) Norwood I procedure with right ventricle to pulmonary artery conduit for hypoplastic left heart syndrome, and (5) arterial switch procedure for transposition of the great arteries. Exclusion criteria included (1) significant pulmonary artery narrowing not addressed surgically, (2) previous pulmonary artery stent placement, (3) previous pulmonary artery angioplasty, (4) significant left-sided atrioventricular valve regurgitation, (5) pulmonary venous return abnormalities, and (6) pulmonary vein stenosis.

Informed written consent was obtained from parents at the preoperative evaluation. One of 3 cardiac surgeons at Vanderbilt Children's Hospital performed the surgical procedures using similar cardiopulmonary bypass and cardioplegia preparations.

Pulmonary hypertension was defined as mean pulmonary arterial pressures of at least 25 mm Hg or exceeding 50% of the mean systemic artery pressure.[10,11]

Pulmonary pressures were also estimated by Doppler echocardiography with the following diagnostic criteria: (1) significant tricuspid regurgitation, (2) enlarged or hypertrophied right ventricle without evidence of pulmonary stenosis, or (3) intraventricular septal flattening.[12,13]

All patients underwent perioperative and postoperative transesophageal echocardiograms per cardiac intensive care protocol. In addition, patients without direct pulmonary arterial monitoring underwent subsequent echocardiograms in the 48-hour postoperative period when clinically indicated for pulmonary hypertension.

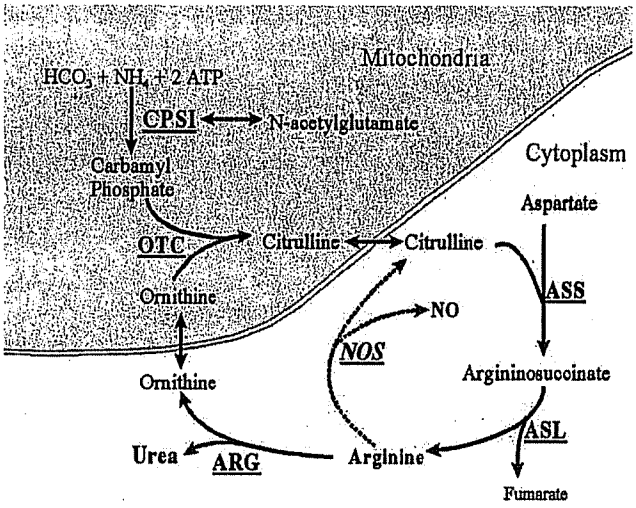

Figure 1. Urea cycle enzymes are differentially found in 2 compartments: the mitochondria of hepatocytes and the cytosol of all cells. Citrulline easily moves across cellular membranes. Once within the cytosol, citrulline is metabolized into L-arginine, which is then metabolized either by arginase into urea or by nitric oxide synthetase into NO. *ATP*, Adenosine triphosphate; *CPSI*, carbamoyl phosphate synthetase I; *OTC*, ornithine transcarbamylase; *ARG*, arginine; *NOS*, nitric oxide synthetase; *NO*, nitric oxide; *ASS*, argininosuccinate synthetase; *ASL*, argininosuccinate lyase.

Echocardiograms were not obtained for pulmonary pressure determination in patients undergoing the modified Fontan or bidirectional Glenn procedures. Perioperative or postoperative transesophageal or transthoracic echocardiograms were interpreted by pediatric cardiologists at Vanderbilt Children's Hospital.

Physicians, nursing staff, and patients/families were blinded to treatment arm assignments. Clinical data and patient demographics were obtained from medical records before knowledge of study results.

Adverse Event

Systemic blood pressure was monitored continuously during the 48-hour study period because of the theoretic risk of hypotension associated with citrulline administration. An adverse event was defined as a greater than 25% decrease in systemic mean blood pressure from baseline (measured before cardiopulmonary bypass). Hypotensive patients were treated with volume resuscitation and/or pharmacologic support. Patients were not withdrawn from the study unless hypotension was unresponsive to interventions.

Study Protocol

Forty patients were randomized to receive either placebo or citrulline perioperatively. Randomization was performed by the Investigational Drug Service of the Vanderbilt Hospital Clinical Pharmacy using computer-generated random numbers in permuted blocks of 4. Patients were enrolled with the intention-to-treat model.

CHD

Citrulline was administered as a 100 mg/mL (10%) solution with distilled water as a suspending agent. The drug and placebo were mixed and distributed by the Investigational Drug Service. Citrulline and placebo were matched for volume and color. Citrulline was administered in 5 doses of 1.9 g/m^2 given every 12 hours for a daily dose of 3.8 g/m^2 and for a total dose of 9.5 g/m^2. This dose was determined by current citrulline replacement therapy for infants and children with urea cycle defects.[14,15]

The first dose of placebo/citrulline was administered through an orogastric feeding tube placed by the research nurse or physician after induction of anesthesia and intubation but before cardiopulmonary bypass in the operating room. The second dose was given immediately on arrival in the pediatric critical care unit (PCCU) for recovery. The third, fourth, and fifth doses were administered at 12, 24, and 36 hours postoperatively in the PCCU, respectively. Postoperative doses were given enterally through a nasogastric feeding tube positioned by the bedside nurse in the PCCU, or by mouth once the patient was extubated.

Sample Collection

Three milliliters of blood were obtained from each patient at 5 time points: immediately before and after bypass, and at 12, 24, and 48 hours postoperatively. The preoperative blood sample was collected after both anesthetic induction and placement of an arterial or a central venous catheter but before surgical incision and study drug administration. The immediate postoperative sample was collected on arrival in the PCCU, and subsequent samples were collected at respective time intervals and before study drug administration. Samples were collected in citrated tubes, placed on ice, and stored at 4°C until processing. Samples were centrifuged within 3 hours of collection for separation of plasma and cellular components. Plasma samples were frozen at −70°C until further laboratory analysis.

Patients were monitored in the PCCU during drug administration and blood collection up to 48 hours postoperatively. If patients had successful recovery before 48 hours with transfer out of the PCCU, central and arterial lines were removed, and therefore drug administration and blood collection were concluded.

Laboratory Measurements

Concentrations of plasma citrulline, arginine, and all other amino acids were determined by amino-acid analysis on protein-free extracts. Amino acids were separated by cation-exchange chromatography using a Hitachi L8800 amino acid analyzer (Hitachi USA, San Jose, Calif). Calibration of the analyzer was completed before testing of patient samples.

Statistical Analysis

Continuous outcome variables, when not normally distributed, were reported as medians with interquartile range (IQR). The Shapiro-Wilk test assessed normality. The Mann-Whitney *U* and Wilcoxon signed-rank tests compared unpaired and paired continuous variables not normally distributed between groups. Dichotomous outcomes for success of randomization and the presence of pulmonary hypertension were reported as proportions and assessed with the Fisher exact test. Parametric testing was used when data were normally distributed and reported as means ± standard deviation. Analysis of covariance assessed differences between groups in repeated measurements of systemic mean blood pressure. All analyses were 2-sided. Statistical analysis was performed with STATA software, version 8.0 (College Station, Tex).

The power calculation for this study was based on the previously reported mean citrulline concentration of 20.7 ± 13.0 μmol/L in children after cardiopulmonary bypass.[4] A sample size of 40 patients equally distributed would have a power (1-β) of 87% to detect a 13 μmol/L (1 standard deviation) difference between oral citrulline (n = 20) and placebo (n = 20) using 2-sided significance and an $\alpha = 0.05$.

TABLE 1. Demographic characteristics between groups

	Placebo (n = 20)	Citrulline (n = 20)	*P*-value
Age-months (median, IQR)	8 (4-29)	12 (0.3-29)	.892
Gender			.751
Male	12 (60%)	10 (50%)	
Female	8 (40%)	10 (50%)	
Ethnicity			1.000
Caucasian	18 (90%)	18 (90%)	
Non-Caucasian	2 (10%)	2 (10%)	
Diagnosis			.901
Single ventricle	11 (55%)	13 (65%)	
VSD/AVSD	6 (30%)	4 (20%)	
TGA	3 (15%)	3 (15%)	
Surgery			.452
BDG/Fontan	11 (55%)	3 (15%)	
VSD/AVSD	6 (30%)	10 (50%)	
Arterial switch	3 (15%)	4 (20%)	
Norwood	0 (0%)	3 (15%)	
Trisomy 21			.661
Present	4 (20%)	2 (10%)	
Absent	16 (80%)	18 (90%)	
Bypass time-minutes (mean ± SD)	112 ± 42	121 ± 47	.520

Results

Patient Enrollment

Forty patients were randomized to receive citrulline (n = 20) or placebo (n = 20). Baseline characteristics between the 2 groups are shown in Table 1. The median age of the study population (N = 40) was 8.5 months (IQR 4-29 months); 55% were male, and 90% were white. Surgical interventions included ventriculoseptal defect or atrioventricular septal defect repair (25%), bidirectional Glenn or Fontan procedures (53%), arterial switch repair for transposition of the great arteries (15%), and Norwood stage I repair (8%).

Safety

Mean blood pressure did not differ between the citrulline and placebo groups ($P = .530$) (Figure 2). No deaths occurred within the 48-hour study period. Three patients died of postoperative complications within 30 days of surgical re-

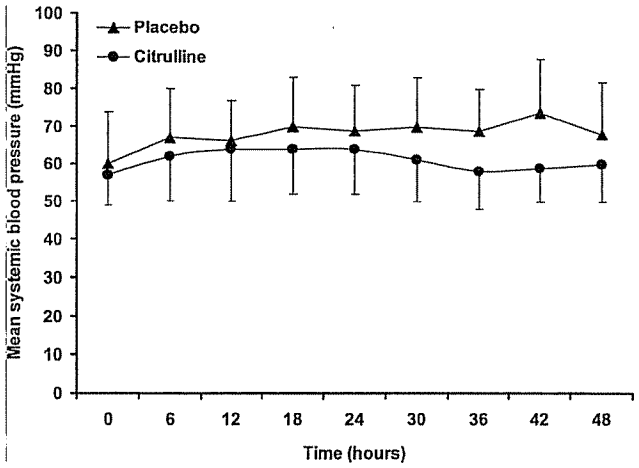

Figure 2. Mean blood pressure did not differ between the oral citrulline and placebo groups (P = .53, multivariate analysis of covariance) throughout the 48-hour study period. Means ± standard deviation are shown for each group.

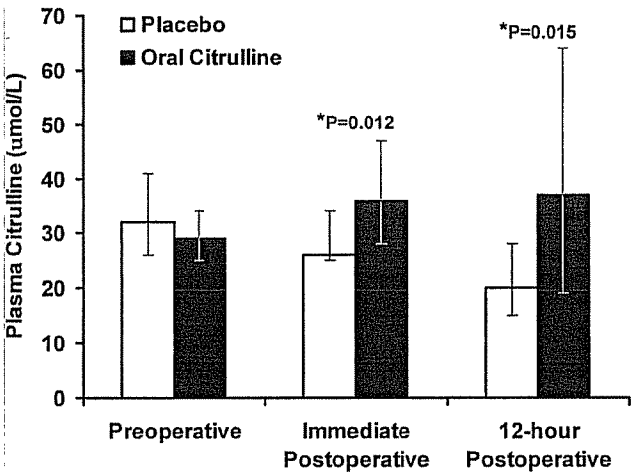

Figure 3. Median plasma citrulline concentrations were significantly higher in the oral citrulline group when compared with the placebo group immediately postoperatively (P = .012) and 12 hours postoperatively (P = .015). The placebo group demonstrated a decrease in citrulline concentrations immediately postoperatively and 12 hours postoperatively (P = .020, P = .001). Medians and 95% confidence intervals are shown for each group.

pair (2 in the citrulline group vs 1 in the placebo group). All deaths were reported to the institutional review board and were found to be unrelated to study drug administration.

All patients (N = 40) completed the study protocol through 12 hours postoperatively. Nine children (4/20 in the citrulline group and 5/20 in the placebo group) successfully recovered by 24 hours postoperatively and transferred out of the PCCU, at which time the study concluded for those individual patients. One patient in the oral citrulline group was withdrawn at 4 hours postoperatively because of complications requiring further surgical intervention, although monitoring continued through 48 hours postoperatively. These patients were represented in all analyses when appropriate through 12 hours postoperatively.

Plasma Citrulline Concentrations

Median plasma citrulline concentrations were no different between groups at baseline (P = .355). After citrulline supplementation, plasma citrulline concentrations were significantly higher in the oral citrulline group when compared with placebo immediately postoperatively (36 μmol/L IQR 28-48 μmol/L vs 26 μmol/L IQR 24-35 μmol/L, P = .012) and 12 hours postoperatively (37 μmol/L IQR 18-83 μmol/L vs 20 μmol/L IQR 15-29 μmol/L, P = .015) (Figure 3). The placebo group demonstrated a significant decrease from baseline in plasma citrulline concentrations after cardiopulmonary bypass immediate postoperatively and 12 hours postoperatively (32 μmol/L IQR 25-44 μmol/L vs 26 μmol/L and 20 μmol/L, P = .020 and P < .001, respectively). In contrast, the oral citrulline group demonstrated a significant increase from baseline in plasma citrulline concentrations by 12 hours postoperatively (29 μmol/L IQR 25-34 μmol/L vs 36 μmol/L, P = .014).

Plasma Arginine Concentrations

Mean plasma arginine concentrations were no different between groups at baseline (P = .495). After citrulline supplementation, plasma arginine concentrations were significantly higher in the oral citrulline group compared with the placebo group by 12 hours postoperatively (36 ± 24 μmol/L vs 23 ± 13 μmol/L, P = .037) (Figure 4). The placebo group demonstrated a significant decrease from baseline in plasma arginine concentrations by 12 hours postoperatively (38 μmol/L IQR 30-52 μmol/L vs 23 μmol/L, P < .001). In contrast, the oral citrulline group maintained postoperative plasma arginine concentrations at baseline through 12 hours postoperatively (33 μmol/L IQR 25-54 μmol/L vs 36 μmol/L, P = .533).

Pulmonary Hypertension

Twenty-eight patients had direct pulmonary arterial pressure measurements (Table 2), of whom 13 of 28 (46%) were in the citrulline group. Pulmonary pressures trended lower in the citrulline group compared with placebo immediately postoperatively (18 ± 5 vs 20 ± 4, P = .127), 6 hours postoperatively (17 ± 5 vs 18 ± 4, P = .290), and 12 hours postoperatively (17 ± 6 vs 17 ± 5, P = .486), although statistical significance could not be demonstrated in this pilot study.

Postoperative pulmonary hypertension developed in 9 patients, 6 of 20 (30%) in the placebo group and 3 of 20

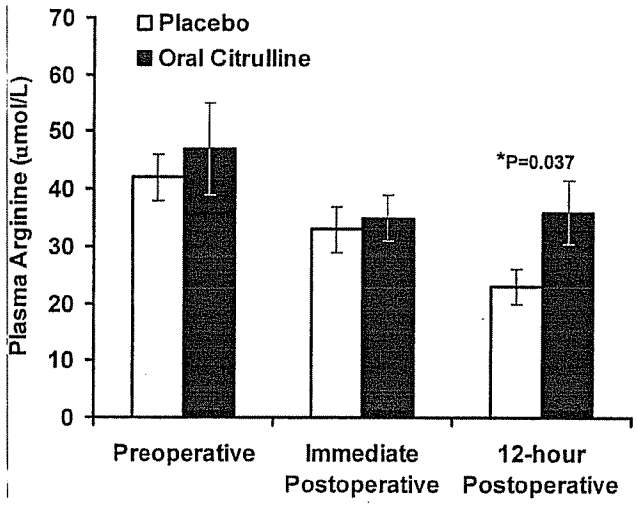

Figure 4. Mean plasma arginine concentrations were significantly higher in the oral citrulline group when compared with the placebo group by 12 hours postoperatively ($P = .037$). The placebo group demonstrated a decrease in plasma arginine concentrations by 12 hours postoperatively ($P < .001$). Means and standard error of mean are shown for each group.

(15%) in the citrulline group ($P = .451$), all of whom had plasma citrulline concentrations less than age-specific norms for children aged less than 6 years (30 μmol/L IQR 23-37 μmol/L).[16] The surgical interventions of these 9 patients included ventriculoseptal defect repair (2/9), atrioventriculoseptal defect repair (1/9), arterial switch repair (3/9), Glenn procedure (1/9), and Fontan procedure (2/9). Patients with pulmonary hypertension had significantly longer cardiopulmonary bypass runs ($P = .04$), significantly longer crossclamp times ($P = .034$), and significantly more postoperative blood loss ($P < .001$) when compared with the study population. Postoperative pulmonary hypertension did not develop in patients with plasma citrulline concentrations in excess of 37 μmol/L ($P = .036$, Table 3).

The mean arginine concentrations were no different between patients with pulmonary hypertension receiving citrulline compared with placebo immediately postoperatively (28 ± 18 μmol/L vs 35 ± 15 μmol/L, $P = .269$) or at other time points. Although the mean citrulline concentrations were significantly higher in patients with pulmonary hypertension receiving citrulline compared with placebo immediately postoperatively (42 ± 21 μmol/L vs 21 ± 8 μmol/L, $P = .025$), concentrations were not significantly different at other time points (Table 4).

Discussion

We found that oral citrulline supplementation safely increased postoperative plasma citrulline and arginine concentrations compared with placebo in children after congenital heart surgery. Mean systemic blood pressure was not different between the oral citrulline and placebo groups, despite significantly different plasma citrulline concentrations. Furthermore, increased plasma citrulline concentrations were associated with a decreased risk of postoperative pulmonary hypertension.

Pulmonary hypertension can be a significant complication in children after surgical correction of their congenital heart lesions.[4] Rescue therapy of postoperative pulmonary hypertension is limited to inhaled NO. Although efficacious, NO is expensive, restricted to inhaled administration, and complicated by rebound pulmonary hypertension after discontinuation.[17] Developments of other therapies such as citrulline supplementation that are safe, inexpensive, and easy to administer will potentially improve postoperative outcomes.

The surgical interventions required for correction of congenital heart defects impair the production of endogenous NO, which disables pulmonary vascular homeostasis. Cardiopulmonary bypass causes pulmonary endothelial dysfunction through reduction of citrulline and arginine substrate, injury to cells secondary to complement activation and effects of oxygen free-radicals, and activation of NO synthase antagonists.[5,6,18] We previously showed that cardiopulmonary bypass leads to significant decreases in both citrulline and arginine concentrations postoperatively, which do not return to baseline even by 48 hours postoperatively.[4] The inability to produce endogenous NO secondary to citrulline and arginine losses in addition to dysfunction of the pulmonary endothelium may exacerbate the risk of postoperative pulmonary hypertension.

Increased pulmonary blood flow, observed with transposition of the great arteries and ventriculoseptal defects, promotes development of pulmonary hypertension through structural and functional abnormalities in the pulmonary vasculature.[19] Other surgical interventions such as the Fontan and bidirectional Glenn procedures rely heavily on low pulmonary pressures for passive pulmonary blood flow. Both the sustained increased preoperative pulmonary blood flow and the deleterious effects of cardiopulmonary bypass are major contributors to the development of postoperative pulmonary hypertension.[5]

The expected significant decrease in plasma citrulline and arginine concentrations after cardiopulmonary bypass was prevented with the administration of oral citrulline. This effect may be secondary to both continuous production of L-arginine from citrulline and stimulation of the NO pathway in both hepatic and pulmonary tissues. Oral citrulline exhibits good bioavailability with ease of movement across cellular membranes. The cytosolic portion of the urea cycle enables localized, intracellular production of L-arginine from citrulline within the pulmonary endothelium.[20] The

TABLE 2. Patients with pulmonary artery pressure measurements after bypass, placebo (cit = 0) and citrulline (cit = 1)

Case	Citrulline	Procedure	Presence of PHTN	Pulmonary arterial pressures (mm Hg)								
				Postoperative	6 h	12 h	18 h	24 h	30 h	46 h	42 h	48 h
4	0	BDG/Fontan	No	19	12	15	16					
6	0	BDG/Fontan	No	15	19	17	17	16	18	17	19	19
8	0	BDG/Fontan	No	12	15	9	12	18				
11	0	BDG/Fontan	Yes	20	16	15	20	18	20	19	17	20
15	0	BDG/Fontan	No	19	17	15	17	17	18	16	17	14
24	0	BDG/Fontan	No	17	18	17	25	21				
27	0	BDG/Fontan	No	18	19	14	13	11	11	8		
28	0	BDG/Fontan	No	19	15	15	18	13	13		16	17
29	0	BDG/Fontan	No	26	19	15	21	15	18	19	22	21
37	0	BDG/Fontan	No	18	19	17	18	18	18	18	22	18
40	0	BDG/Fontan	No	18	12							
10	0	VSD/AVSD	No	25								
16	0	VSD/AVSD	Yes	22	16	21	21	19	23	28	19	26
35	0	VSD/AVSD	Yes	25	26							
2	0	Switch	Yes	23	27	31	32	29	31	30	34	30
1	1	BDG/Fontan	No	12	10	9	10	9				
5	1	BDG/Fontan	No	16	17	10	14	16	11	2	11	
7	1	BDG/Fontan	Yes	21	19	22	29	22	27	22	35	30
9	1	BDG/Fontan	No	22	15		18	18	21	16	12	14
13	1	BDG/Fontan	No	16	13	12	9	12				
14	1	BDG/Fontan	No	6	13	17	6					
25	1	BDG/Fontan	No	24	9	17	15	15	14	17	15	19
26	1	BDG/Fontan	No	16	16	12						
38	1	BDG/Fontan	No	17	16	15	17					
39	1	BDG/Fontan	Yes	19	25	31	22	20	20	23	23	21
20	1	VSD/AVSD	No	21	20	17						
21	1	VSD/AVSD	No	17	22	18	20					
3	1	Switch	Yes	24	24	20	25	25	23	23	29	28

BDG, Bidirectional Glenn; *VSD,* ventriculoseptal defect; *AVSD,* atrioventricular septal defect; *PHTN,* pulmonary hypertension. Pulmonary hypertension was defined as mean pulmonary arterial pressures (MPAP) of at least 25 mmHg and/or exceeding 50% of the mean systemic artery pressure (MSAP).[10,11] The citrulline group had PA pressures which trended lower than the placebo group immediately postoperative (18 ± 5 vs 20 ± 4, $P = .127$), 6-hours postoperative (17 ± 5 vs 18 ± 4, $P = .290$), and 12-hours postoperative (17 ± 6 vs 17 ± 5, $P = .486$), although statistical significance could not be demonstrated in this pilot study.

TABLE 3. Low risk of pulmonary hypertension with high plasma citrulline

Plasma citrulline 12 h postoperatively	Pulmonary hypertension absent	Pulmonary hypertension present	*P* value
<37 μmol/L	18	9	
≥37 μmol/L	12	*0	.036

local production of NO may be inferred from the association of high plasma citrulline concentrations and the decreased incidence of pulmonary hypertension without coexisting systemic hypotension as suggested by this study.

The risk and severity of postoperative pulmonary hypertension may vary on the basis of the type of congenital heart defect or surgical intervention required. We did not restrict enrollment to a single cardiac lesion/surgical intervention, despite the possible concern for confounders. Restricting enrollment to a homogenous population would not have appropriately addressed the treatment issues associated with the complicated disease process of pulmonary hypertension. Larger intervention trials can stratify randomization by diagnosis that may decrease concern for confounders. We randomized patients equally between the oral citrulline and placebo groups without stratification because of the small sample size. Consequently, all 3 patients undergoing the Norwood I procedure were randomly assigned to the citrulline group. This imbalance had a risk of favoring the null hypothesis because these patients historically have more complicated postoperative courses associated with extremely low cardiac output states. However, these patients demonstrated a relatively uncomplicated 48-hour postoperative course, which presumably permitted adequate absorption of citrulline and appropriate assessment of its clinical effects.

TABLE 4. Characteristics of patients with pulmonary hypertension diagnosis

Case	Citrulline	Age	Procedure	Method of PHTN diagnosis		Plasma arginine μmol/L					Plasma citrulline μmol/L				
				Echo	PAP	Pre	Post	12 h	24 h	48 h	Pre	Post	12 h	24 h	48 h
2	Placebo	<1 mo	Switch		Yes	104	52	24	13	56	15	9	7	5	4
31	Placebo	<1 mo	Switch	Yes		56	36	44	45	213	23	12	10	9	17
16	Placebo	4 mo	AVSD		Yes	39	8	13	5	4	47	26	31		13
35	Placebo	5 mo	VSD	Yes		70	45	33	27	33	40	24	28	16	10
36	Placebo	8 mo	VSD	Yes		22	37	5	6	4	18	24	12	11	12
11	Placebo	8 mo	BDG		Yes	30	34	13	19	11	41	29	29	26	16
3	Citrulline	<1 mo	Switch		Yes	24	41	11	8	18	27	66	17	15	17
7	Citrulline	29 mo	Fontan		Yes	57	36	79			36	29	19		
39	Citrulline	25 mo	Fontan		Yes	33	7	15	13	8	24	32	18	6	6

PHTN, Pulmonary hypertension; *Echo,* echocardiography; *PAP,* pulmonary arterial pressure; *AVSD,* atrioventricular septal defect; *VSD,* ventriculoseptal defect; *BDG,* bidirectional Glenn. The median citrulline concentration was significantly higher in the citrulline group versus placebo immediately postoperative (36 umol/L vs 26 umol/L, $P = .012$) and at 12-hours postoperative (37 umol/L vs 20 umol/L, $P = .015$). *Patients with plasma citrulline concentrations in excess of 37 umol/L, whether naturally occurring or with citrulline supplementation, did not develop postoperative pulmonary hypertension ($P = .036$).

This pilot study demonstrated that an oral citrulline regimen used in children with urea cycle defects can also be safely administered to children undergoing cardiopulmonary bypass with subsequent elevations in plasma citrulline concentrations. We were able to further demonstrate an association between citrulline concentrations and occurrence of postoperative pulmonary hypertension. Two limitations in definitively describing this relationship included the method of pulmonary hypertension diagnosis and the citrulline-dosing regimen. Our study protocol limited interference of routine postoperative care, and therefore pulmonary arterial lines were not mandated. Diagnosis of postoperative pulmonary hypertension in patients without direct pulmonary pressure measurement was completed by echocardiography, which has known limitations.

Few patients receiving oral citrulline did not demonstrate the significant increase in plasma citrulline concentration, and consequently were at risk for postoperative pulmonary hypertension. Risk factors for development of pulmonary hypertension in this study population included significantly longer cardiopulmonary bypass runs, longer crossclamp times, and more postoperative blood loss. Inability to overcome pulmonary endothelial dysfunction because of ongoing citrulline losses after bypass may have exacerbated the risk of developing pulmonary hypertension in these patients. We expect that higher doses of oral citrulline will achieve levels in much greater excess than 37 μmol/L, and that with consistently elevated citrulline concentrations pulmonary hypertension may be prevented. With this pilot study, we targeted those plasma concentrations that are associated with a decreased risk of pulmonary hypertension. Furthermore, the safety data and plasma concentrations achieved with oral citrulline enabled us to begin pharmacokinetic studies of intravenous citrulline and its future use in the treatment of pulmonary hypertension.

Conclusion

Oral citrulline supplementation safely increased plasma concentrations of both citrulline and arginine compared with placebo. Elevations in plasma citrulline concentrations above age-specific norms, whether naturally occurring or with citrulline supplementation, were associated with a decreased risk of postoperative pulmonary hypertension. Therefore, aggressive oral citrulline supplementation or future use of intravenous citrulline, resulting in consistent citrulline concentrations in excess of 37 μmol/L, may prevent postoperative pulmonary hypertension.[7]

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CHD

Pharmacokinetics and safety of intravenously administered citrulline in children undergoing congenital heart surgery: Potential therapy for postoperative pulmonary hypertension

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Objective: Pulmonary hypertension may complicate surgical correction of congenital heart defects, resulting in increased morbidity and mortality. We have previously shown that plasma levels of the nitric oxide precursors citrulline and arginine drop precipitously after congenital cardiac surgery and that oral citrulline supplementation may be protective against the development of pulmonary hypertension. In this study, we assessed the safety and pharmacokinetic profile of intravenous citrulline as a potential therapy for postoperative pulmonary hypertension.

Methods: The initial phase of this investigation was a dose-escalation study of intravenously administered citrulline in infants and children undergoing one of five congenital cardiac surgical procedures (phase 1). The primary safety outcome was a 20% drop in mean arterial blood pressure from the baseline pressure recorded after admission to the intensive care unit. Based on our previous work, the target circulating plasma citrulline trough was 80 to 100 μmol/L. Each patient was given two separate doses of citrulline: the first in the operating room immediately after initiation of cardiopulmonary bypass and the second 4 hours later in the pediatric intensive care unit. Stepwise dose escalations included 50 mg/kg, 100 mg/kg, and 150 mg/kg. After model-dependent pharmacokinetic analysis, we enrolled an additional 9 patients (phase 2) in an optimized dosing protocol that replaced the postoperative dose with a continuous infusion of citrulline at 9 mg/(kg · h) for 48 hours postoperatively.

Results: The initial stepwise escalation protocol (phase 1) revealed that an intravenous citrulline dose of 150 mg/kg given after initiation of cardiopulmonary bypass yielded a trough level of in the target range of approximately 80 to 100 μmol/L 4 hours later. The postoperative dose revealed that the clearance of intravenously administered citrulline was 0.6 L/(h · kg), with a volume of distribution of 0.9 L/kg and estimated half-life of 60 minutes. Because of the short half-life, we altered the protocol to replace the postoperative dose with a continuous infusion of 9 mg/(kg · h). An additional 9 patients were studied with this continuous infusion protocol (phase 2). Mean plasma citrulline levels were maintained at approximately 125 μmol/L, with a calculated clearance of 0.52 L/(h · kg). None of the 17 patients studied had a 20% drop in mean arterial blood pressure from baseline.

Conclusions: In this first report of the use of intravenous citrulline in humans, we found citrulline to be both safe and well tolerated in infants and young children undergoing congenital cardiac surgery. Because of the rapid clearance, the optimal dosing regimen was identified as an initial bolus of 150 mg/kg given at the initiation of cardiopulmonary bypass, followed 4 hours later by a postoperative infusion of 9 mg/(kg · h) continued up to 48 hours. Using this regimen, plasma arginine, citrulline, and nitric oxide metabolite levels were well maintained. Intravenous citrulline needs to be studied further as a potential therapy for postoperative pulmonary hypertension.

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Pulmonary hypertension is a potential complication after surgical correction of congenital cardiac defects that has been associated with increased postoperative morbidity and mortality.[1-5] Current perioperative treatment includes the use of inhaled nitric oxide and several nonselective pulmonary vasodilators, including milrinone, epoprostenol, sildenafil citrate (INN sildenafil), and generation of alkalosis. Another potential therapy includes increasing endogenous nitric oxide synthesis by supplementation with citrulline or arginine.[6,7] Nitric oxide is produced from L-citrulline and L-arginine, amino acids generated through the urea cycle (Figure 1).[8-10] We have previously demonstrated that citrulline and arginine levels drop precipitously after surgical correction of congenital cardiac defects with cardiopulmonary bypass and do not recover for as long as 48 hours after surgery.[11] In addition, children with postoperative pulmonary hypertension had significantly lower plasma arginine levels than those without this complication. We subsequently conducted a randomized, placebo-controlled trial of oral citrulline supplementation in this same patient population and found that although absorption was variable, citrulline was safe and that children who had a plasma citrulline level greater than 40 μmol/L at 12 hours after surgery were free from postoperative pulmonary hypertension.[10]

Intravenously administered citrulline has not been previously studied in human beings. The hypothesis of this study was that intravenously administered citrulline would be safe and would prevent the postoperative drop in plasma citrulline and arginine levels noted in our previous observational studies.

Materials and Methods

Patient Enrollment

Approval from Vanderbilt's institutional review board (IRB) was obtained before patient enrollment. A total of 17 patients were enrolled in this open-label dose-escalation study at Vanderbilt Children's Hospital between May 2005 and January 2006.

All infants or children younger than 6 years undergoing one of five surgical procedures for correction of congenital heart lesions were considered for enrollment. The eligible surgical procedures were as follows: (1) repair of atrioventricular septal defect, (2) repair of ventricular septal defect, (3) bidirectional Glenn procedure (superior cavopulmonary shunt), (4) modified Fontan procedure (total cavopulmonary connection), and (5) arterial switch procedure. Exclusion criteria were as follows: (1) significant pulmonary arterial narrowing not addressed surgically, (2) previous pulmonary artery stent placement, (3) previous pulmonary artery angioplasty, (4) significant left-sided atrioventricular valve regurgitation, (5) pulmonary venous return abnormalities, (6) pulmonary vein stenosis, (7) preoperative mechanical ventilation, and (8) preoperative inotropic infusions.

Informed written consent was obtained from parents of the enrolled patients during preoperative evaluation at the Cardiothoracic Surgery Clinic (outpatient) or at Vanderbilt Children's Hospital (inpatient). Three cardiac surgeons at Vanderbilt Children's Hospital (K.G.C., D.C.D., F.S.) performed the surgical procedures with similar cardiopulmonary bypass and cardioplegia preparations.

This study was monitored closely by a data safety monitoring board (DSMB) composed of a pediatric cardiologist, a pediatric critical care physician, and a general pediatrician. The DSMB met three times during the study to review the safety and pharmacokinetic data.

Abbreviations and Acronyms

DSMB = data safety monitoring board
FDA = Food and Drug Administration
IRB = institutional review board
k_{rem} = constant of citrulline removal
PICU = pediatric intensive care unit
R_{app} = rate of citrulline appearance

Adverse Events

Intravenous citrulline administration carries a theoretic risk of systemic arterial hypotension. An adverse drop in mean arterial pressure was defined as a decrease of more than 20% from baseline. The baseline postoperative mean arterial blood pressure was calculated as the average of mean arterial blood pressure measurements collected every 5 minutes for the 30 minutes immediately before the administration of the postoperative dose or infusion. The bedside monitor was then set to alarm if that 20% drop was reached at any time in the 48-hour study period. If the bedside monitor reached the preset limit and alarmed, the beside nurse was instructed to alert the study physician or nurse and to record mean arterial pressure every 5 minutes for 30 minutes. If the average of these 5-minute recordings was 20% below the original baseline blood pressure, the citrulline was discontinued. Patients were treated for hypotension at the discretion of the clinical pediatric intensive care unit (PICU) staff with volume resuscitation, inotropic or vasopressor support, or both. Development of hypotension according to these criteria that necessitated discontinuation of citrulline was counted as an adverse event and was reported to the DSMB, IRB, and Food and Drug Administration (FDA).

Serious adverse events, such as cardiac arrest, need for extracorporeal membrane oxygenation, and death, were reported immediately to the DSMB, the Vanderbilt IRB, and the FDA.

Study Protocol

The study design for the first 8 patients (phase 1) was a dose-escalation protocol with two intravenously administered bolus doses of citrulline to determine the optimal dose and characterize pharmacokinetic parameters including half-life, clearance, and volume of distribution. The first bolus (given during the course of 10 minutes) was administered after initiation of cardiopulmonary bypass in the operating room; and the second (given during the course of 30 min) was administered 4 hours later in the critical care unit. The intravenous doses of citrulline for each bolus were 50 mg/kg (2 patients), 100 mg/kg (2 patients), and 150 mg/kg (4 patients).

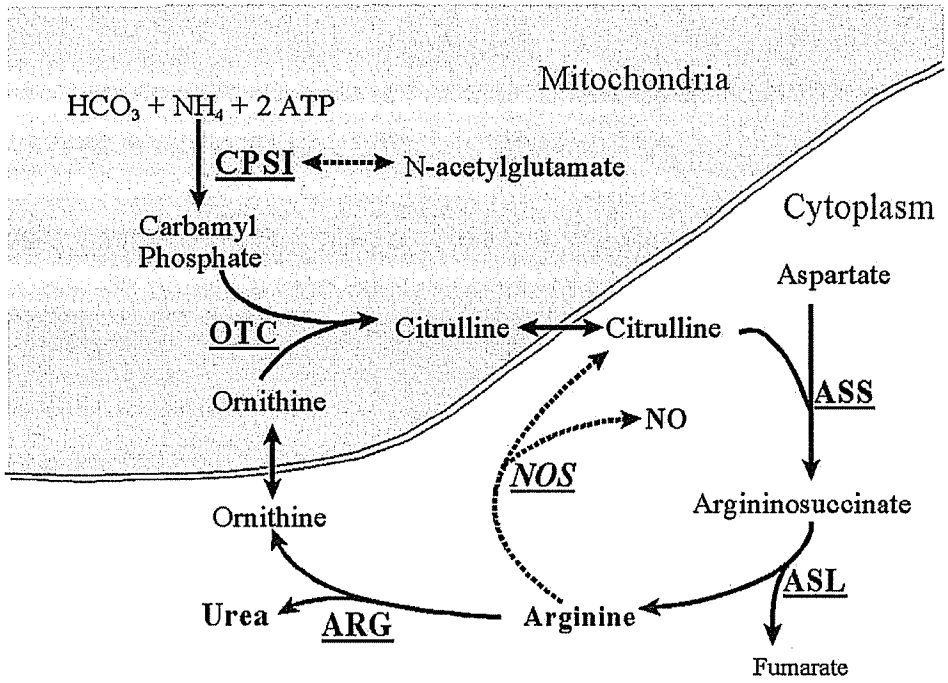

Figure 1. Carbamyl phosphate synthetase I *(CPSI)* is rate-limiting enzyme of urea cycle. Polymorphisms in its gene alter availability of nitric oxide precursors, citrulline, and arginine. Citrulline is metabolized into arginine, which is then metabolized into either nitric oxide *(NO)* by nitric oxide synthetase *(NOS)* or urea by arginase *(ARG)*. HCO_3, Bicarbonate; NH_4, ammonium; *ATP*, adenosine triphosphate; *OTC*, ornithine transcarbamylase; *ASS*, argininosuccinate synthetase; *ASL*, argininosuccinate lyase. From Smith HA, Canter JA, Christian KG, Drinkwater DC, Scholl FG, Christman BW, et al. Nitric oxide precursors and congenital heart surgery: a randomized controlled trial of oral citrulline. *J Thorac Cardiovasc Surg.* 2006;132:58-65.

The study design was changed (phase 2) after it was determined that the half-life and clearance were not compatible with intermittent dosing. After consultation with a pharmacologist, the next 9 patients received a 150-mg/kg intravenously administered bolus of citrulline during the course of 10 min in the operating room after initiation of cardiopulmonary bypass, followed 4 hours later in the critical care unit by a continuous infusion of 9 mg/(kg · h) that continued for 48 hours.

The citrulline preparation was provided by the Investigational Drug Service of the Vanderbilt Hospital Clinical Pharmacy. Citrulline was administered as a 50-mg/mL (5%) isotonic solution, with distilled water as a suspending agent.

Sample Collection

A 3-mL sample of blood was obtained from each patient at selected time points. In phase 1, samples were collected immediately after initiation of cardiopulmonary bypass before the first bolus given in the operating room, postoperatively 4 hours after the operating room bolus (immediately before the postoperative bolus), and then 1, 2, 3, 4, and 12 hours after the postoperative bolus. In phase 2, samples were collected immediately after initiation of cardiopulmonary bypass (before the first bolus given in the operating room), immediately after the operating room bolus, postoperatively 4 hours after the operating room bolus (immediately before the postoperative infusion), and then 6, 12, 24, and 48 hours after initiation of the postoperative continuous infusion. Samples were collected in citrated tubes, placed on ice, and stored at 4°C until processing. Samples were centrifuged for separation of plasma and cellular components. Plasma samples were frozen at −70°C until further laboratory analysis.

Laboratory Measurements

Concentrations of plasma citrulline and arginine were determined through amino acid analysis by cation-exchange chromatography with a Beckman 7300 amino acid analyzer (Beckman Coulter, Inc, Fullerton, Calif). Calibration of the analyzer with known standards was completed before testing of patient samples.

Nitric oxide metabolites were measured by chemiluminescence with a Sievers 280 nitric oxide analyzer (GE Analytical Instruments, Boulder, Colo). Plasma samples were mixed 1:2 sample/cold ethanol at 0°C for 30 minutes. After centrifugation at 14,000 rpm for 5 minutes, samples were injected into the analyzer. This method relies on catalytic reduction of nitric oxide metabolites by exposure to warm vanadium hydrochloride. Liberated nitric oxide was driven by nitrogen gas into an ozone chamber. Light released by nitric oxide–ozone interaction was captured by a photomultiplier tube and relayed to an analytic software program. A standard curve with sodium nitrite was used to determine sample concentrations.

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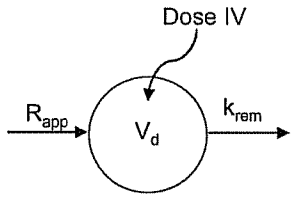

Figure 2. Pharmacokinetic model used in analysis of phase 1 data and for design of phase 2 intravenous *(IV)* dosing regimen. In this model, body is represented as single compartment with volume of distribution (V_d). Citrulline appearance in plasma is described by zero-order process *(R_{app})* to account for endogenous production, whereas removal from plasma is determined by first-order process *(k_{rem})*.

Pharmacokinetic Analysis

Data obtained for each patient in phase 1 were fitted to the pharmacokinetic model depicted in Figure 2. In this model, the body exists as a single compartment with a volume of distribution. The appearance of citrulline in plasma is described by a zero-order process (rate of citrulline appearance, R_{app}) to account for endogenous production, whereas the removal of citrulline is determined by a first-order process (constant of citrulline removal, k_{rem}). It is assumed that the values of all parameters remained constant for each patient during the course of plasma sampling. Mass–balance differential equations were inputted into SCIENTIST (MicroMath Scientific Software, St Louis, Mo) and solved numerically. Data fitting was accomplished by a weighted, least squares procedure to obtain the simultaneous estimates of R_{app}, k_{rem}, and volume of distribution. Clearance was calculated from k_{rem} multiplied by the volume of distribution, and half-life was estimated as $\ln 2/k_{rem}$.

Results

Patient Enrollment

Seventeen patients were successfully enrolled, 8 patients in phase 1 and 9 in phase 2. The median age of the patients was 6 months (interquartile range 3.6–30.6 months), with 55% male and 81% white. Surgical interventions were as follows: 4 patients with ventricular septal defect repair, 8 patients with atrioventricular septal defect repair, 2 patients with bidirectional Glenn shunt, 1 patient with modified Fontan procedure, and 2 patients with arterial switch.

Safety

There were no significant adverse events in phase 1. There was 1 significant adverse event in phase 2. That patient underwent an atrioventricular septal defect repair and was in a junctional rhythm postoperatively, necessitating atrial pacing. At approximately 8 postoperative hours, the patient showed the acute onset of profound bradycardia consistent with complete heart block that was not preceded by systemic hypotension and was not responsive to ventricular pacing. Advanced life-support measures were instituted, including open cardiac massage and emergency cannulation for venoarterial extracorporeal membrane oxygenation, which was required for 48 hours. The patient subsequently recovered fully and was discharged home on hospital day 22. The DSMB reviewed the case and determined that the significant adverse event was unlikely to be related to the citrulline administration. The adverse event was also reported to the Vanderbilt IRB, the National Institutes of Health, and the FDA.

Pharmacokinetics

Phase 1. Patients in phase 1 were given two doses of intravenously administered citrulline, the first just after initiation of cardiopulmonary bypass and the second 4 hours later after admission to the PICU. The plasma levels of citrulline at the various times after drug administration are depicted in Figure 3. Patients 1 and 2 received 50-mg/kg citrulline intravenously and had a peak citrulline level of approximately 220 μmol/L and a 4-hour trough level of 40 μmol/L. No adverse side effects were noted. This trough was well below the target range of 80 to 100 μmol/L, and the dose was subsequently increased. Patients 3 and 4 received 100-mg/kg citrulline intravenously and had a peak citrulline level of 375 μmol/L and a 4-hour trough of 50 μmol/L. Again, no adverse side effects were noted. This trough was also below the target range of 80 to 100 μmol/L, and the dose was subsequently increased. Patient 5, 6, 7, and 8 received 150 mg/kg citrulline intravenously and had a peak citrulline level of 660 μmol/L and a 4-hour trough of 80 μmol/L. This 4-hour trough was in the target range of 80 to 100 μmol/L, and the dose was not escalated further. The citrulline pharmacokinetic parameter estimates for each of the three dosage levels are summarized in Table 1, and the pharmacokinetic profiles are displayed in Figure 3. The half-life was calculated to be approximately 60 minutes, which was too short to proceed with intermittent dosing. The study design was changed to a bolus dose followed by a continuous infusion in phase 2.

Phase 2. An additional 9 patients were enrolled in phase 2. On the basis of parameter estimates obtained from phase 1 (Table 1), pharmacokinetic simulations predicted that a bolus dose of 150 mg/kg followed 4 hours later by a continuous infusion of 9 mg/(kg · h) would yield sustained plasma citrulline levels of approximately 80 to 100 μmol/L (Figure 4). Initiation of the continuous infusion was deliberately set at 4 hours after the bolus to allow sustained increased levels during separation from cardiopulmonary bypass and ultrafiltration, to allow time for admission to the PICU, and to allow postoperative hemodynamic stabilization to allow accurate assessment of the drug safety profile. The phase 2 mean plasma citrulline profile is shown in Figure 5. For the entire group, postoperative mean plasma citrulline levels were sustained at approximately 150 to 250

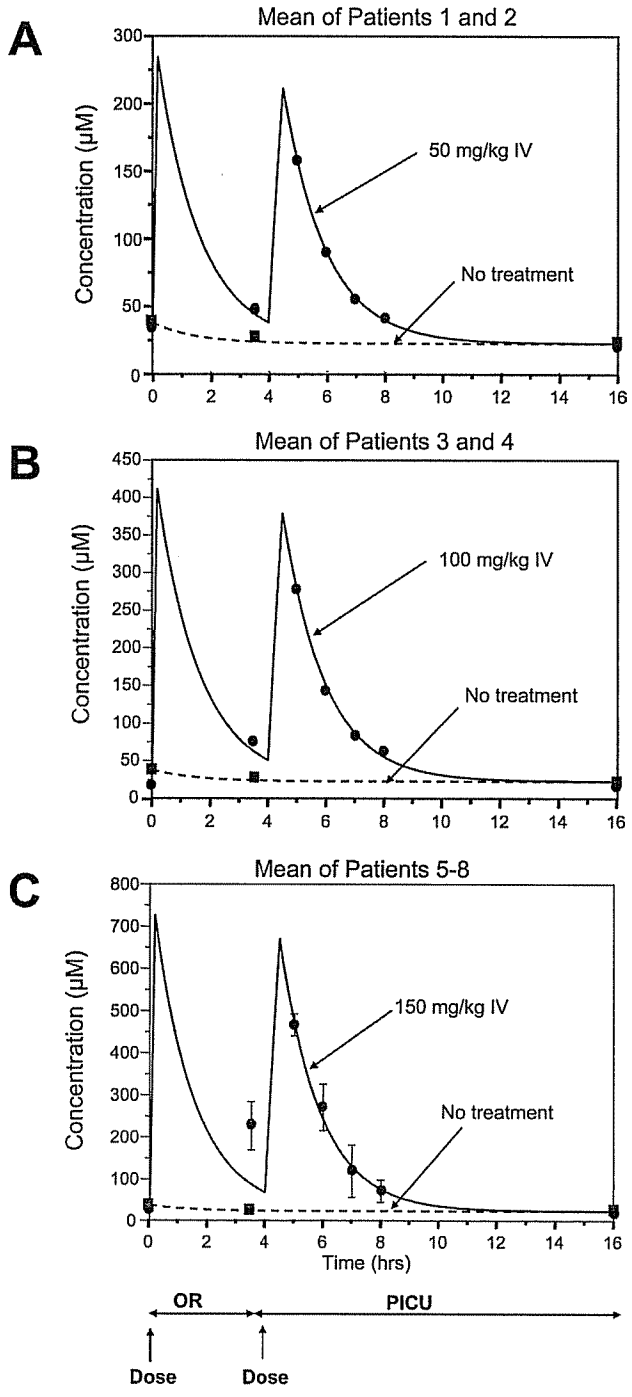

Figure 3. Pharmacokinetic profile of intravenously administered *(IV)* bolus-dose citrulline in phase 1. Dose-escalation study design in which each patient was given two boluses of citrulline, first in operating room *(OR)* on cardiopulmonary bypass and second 4 hours later after admission to pediatric intensive care unit *(PICU)*. Patients 1 and 2 received 50 mg/kg (A), patients 3 and 4 received 100 mg/kg (B), and patients 5, 6, 7, and 8 received 150 mg/kg (C). No adverse side effects were noted.

μmol/L during the 48-hour study period. Estimated citrulline clearance was 0.52 ± 0.28 L/(kg · h) in phase 2. The mean plasma citrulline levels of a previously published cohort of patients undergoing congenital cardiac surgery who had not received intravenously administered citrulline are also depicted in Figure 5 for comparison.[11] Figure 6 depicts similar data for plasma arginine levels, which were also sustained after intravenous administration of citrulline relative to data from the previous cohort.[11] Figure 7 depicts similar data for plasma nitric oxide metabolite levels, which were also sustained after intravenous administration of citrulline relative to data from the previous cohort.[11]

Discussion

This is the first report of the use of intravenously administered citrulline in human beings. The goal of this study was to determine the safety profile and pharmacokinetics of intravenously administered citrulline in a fairly unique patient population of infants and children undergoing surgical correction of congenital heart defects. This was an open-label study that was not placebo controlled and thus not designed to evaluate the use of intravenously administered citrulline as a potential therapy for postoperative pulmonary hypertension. The pharmacokinetic and safety information gained from this study, however, has been subsequently used to design a randomized, placebo-controlled efficacy trial of intravenously administered citrulline for the treatment of postoperative pulmonary hypertension.

With regard to safety, we were primarily interested in systemic hypotension as a potential negative side effect of citrulline treatment. We defined systemic hypotension as a sustained 20% drop in mean arterial pressure from an average baseline recording that was obtained during the 30 minutes just before initiation of intravenous citrulline administration, either as a bolus (phase 1) or as a continuous infusion (phase 2). This definition took into account some of the inherent blood pressure variation in children immediately after congenital cardiac surgery. None of the patients in phase 1 met this definition of systemic hypotension. Only 1 patient in phase 2 of this study met this definition, and that patient's hypotension was precipitated by profound bradycardia and heart block, a side effect unlikely to be precipitated by citrulline. The DSMB reviewed that patient's clinical data and determined that the adverse event was unlikely to be related to the citrulline infusion. The Vanderbilt IRB, the National Institutes of Health, and the FDA were also notified and concurred with this conclusion.

The pharmacokinetics of intravenously administered citrulline in this patient population are significantly complicated by the interposition of cardiopulmonary bypass and ultrafiltration. We initially decided to administer a fairly large bolus of citrulline immediately after initiation of cardiopulmonary bypass, with the goal of providing a 4-hour

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TABLE 1. Model-dependent citrulline pharmacokinetic parameter estimates

	Patients 1 and 2	Patients 3 and 4	Patients 5-8
Dose (mg/kg)	50	100	150
R_{app} (μmol/[h · kg])	19.1 (15.9, 22.2)	14.7 (13.4, 16.0)	10.8 ± 1.9
k_{rem} (h^{-1})	0.78 (0.67, 0.89)	0.72 (0.87, 0.56)	0.68 ± 0.16
Volume of distribution (L/kg)	0.99 (0.97, 1.02)	0.99 (0.77, 1.22)	0.89 ± 0.24
Clearance (L/[h · kg])	0.78 (0.65, 0.91)	0.68 (0.67, 0.8)	0.58 ± 0.05

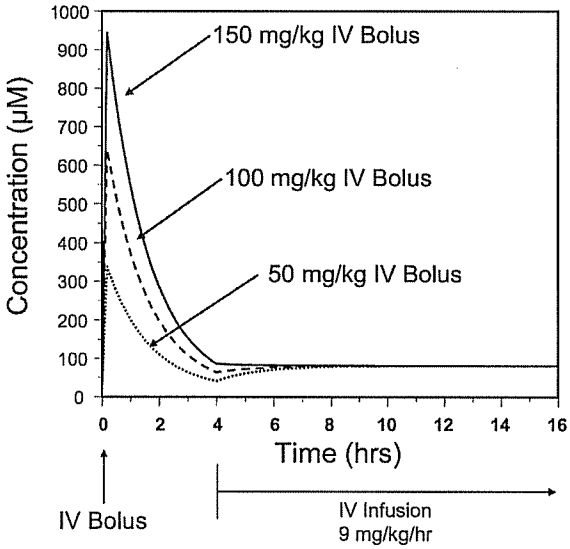
Data represent means of individual values (± SD for patients 5 through 8; individual values are given in parentheses for patients 1 and 2 and patients 3 and 4) of parameter estimates from pharmacokinetic model shown in Figure 2. R_{app}, Rate of citrulline appearance; k_{rem}, constant of citrulline removal.

target trough level of approximately 80 to 100 μmol/L. This target level was intentionally above the threshold value of approximately 40 μmol/L that we had previously identified as potentially protective against postoperative pulmonary hypertension in our studies with orally administered citrulline.[10] With an intravenously administered citrulline bolus of 150 mg/kg, we were able to achieve that target 4-hour trough level. An alternative approach would have been to use a smaller initial bolus followed by an immediate continuous infusion, but the mechanics of cardiopulmonary bypass and ultrafiltration both during and after bypass made this approach impractical.

In phase 1 of the study, we determined that the half-life of intravenously administered citrulline is fairly short at approximately 60 minutes. The volume of distribution of citrulline was estimated between 0.8 and 1.0 L/kg among the dosage groups in Phase 1 (Table 1). This value suggests that citrulline distributes to extravascular spaces. The high postdosing levels observed during phase 2 suggest that citrulline rapidly distributes out of the vascular space. The short half-life of citrulline is largely due to efficient elimination by the body, with clearance 0.6 to 0.8 L/(kg · h).

We did not study intravenously administered citrulline in patients who were not undergoing cardiac surgery. With computer modeling of data obtained from the second postoperative bolus dose in phase 1 of this study, however, we estimated that a bolus of 20 mg/kg immediately followed by a continuous infusion of 9 mg/(kg · h) would rapidly achieve steady state at plasma levels of 100 μmol/L in other patient groups at risk for pulmonary hypertension, in whom the effects of cardiopulmonary bypass would not be a concern in establishing the pharmacokinetics of citrulline. This noncardiac surgical protocol would need to be validated in further studies.

Determination of whether intravenously administered citrulline is a potential therapy for postoperative pulmonary hypertension will require a randomized clinical trial. We have recently initiated a trial of intravenously administered citrulline versus saline placebo according the described pro-



Bolus (mg/kg)	Model Predicted Peak (μM)	Model Predicted Trough (4hrs) (μM)
50	340	42
100	645	64
150	945	87

Figure 4. Pharmacokinetic modeling of intravenously administered *(IV)* bolus of citrulline given at beginning of surgery, followed 4 hours later by continuous infusion. Bolus dose of 150 mg/kg was determined most likely to yield 4-hour trough of 80 to 100 μmol/L, and infusion of 9 mg/(kg · h) was predicted to achieve steady state. This protocol was used in phase 2.

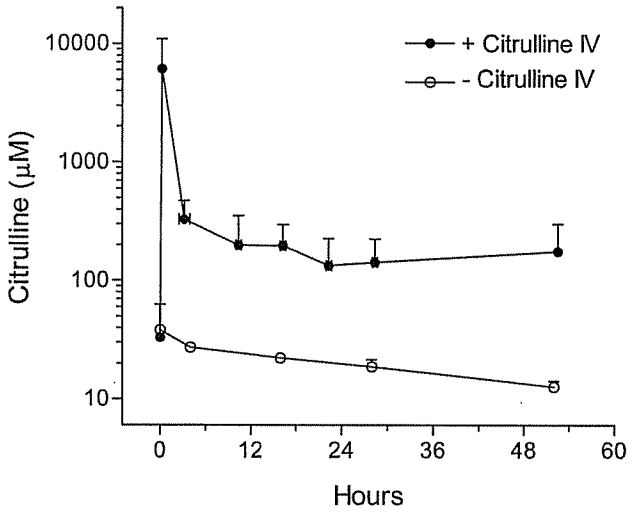


Figure 5. Plasma concentration–time profile of citrulline in phase 2. Patients 9 to 17 were administered citrulline 150 mg/kg intravenously *(IV)* in operating room on cardiopulmonary bypass, followed by continuous infusion at 9 mg/(kg · h) initiated 4 hours later after admission to pediatric intensive care unit. Target citrulline levels were achieved with this citrulline dosing regimen. For comparison, citrulline plasma levels in infants undergoing similar procedures without citrulline treatment in previously published study are shown.[11]

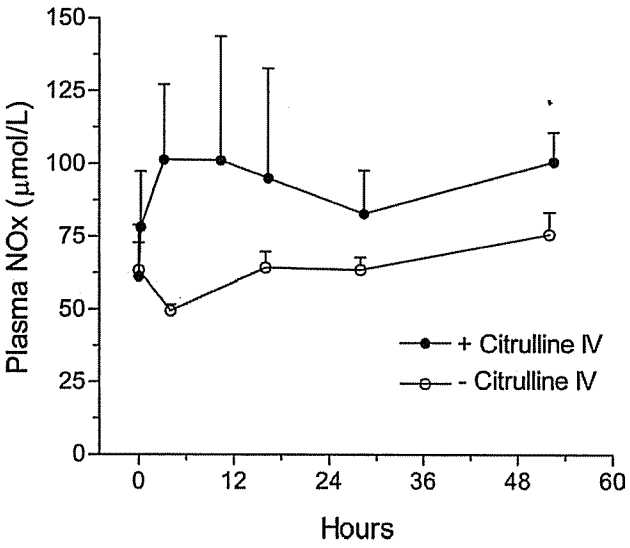


Figure 7. Plasma concentration–time profile of nitric oxide metabolites *(NOx)* in phase 2. Patients 9 to 17 were administered citrulline 150 mg/kg intravenously *(IV)* in operating room on cardiopulmonary bypass, followed by continuous infusion at 9 mg/(kg · h) initiated 4 hours later after admission to pediatric intensive care unit. For comparison, nitric oxide metabolite plasma levels in infants undergoing similar procedures without citrulline treatment in previously published study are shown.[10]

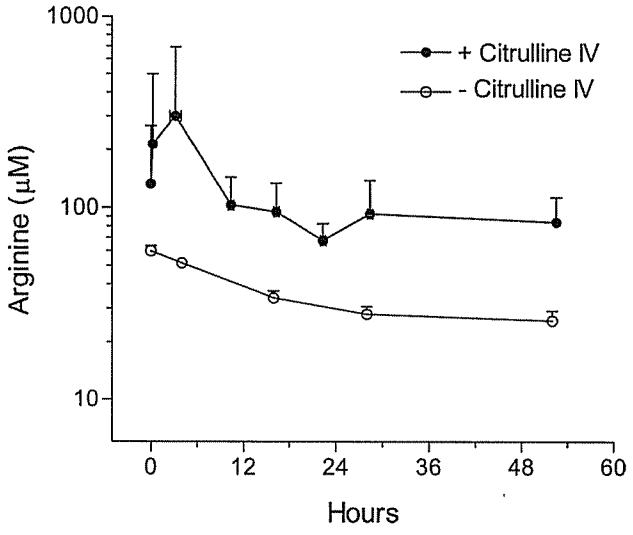


Figure 6. Plasma concentration–time profile of arginine in phase 2. Patients 9 to 17 were administered citrulline 150 mg/kg intravenously *(IV)* in operating room on cardiopulmonary bypass, followed by continuous infusion at 9 mg/(kg · h) initiated 4 hours later after admission to pediatric intensive care unit. For comparison, arginine plasma levels in infants undergoing similar procedures without citrulline treatment in previously published study are shown.[11]

tocol in infants and children undergoing these same five cardiac surgical procedures, with a goal of reducing the incidence of postoperative pulmonary hypertension by 50%. In addition, the efficacy of combination therapy of orally or intravenously administered citrulline and other therapies for pulmonary hypertension, such as inhaled nitric oxide, nebulized inhaled epoprostenol, and orally administered sildenafil citrate, should be investigated.

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Exhibit C

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Exhibit C

L-Citrulline ameliorates chronic hypoxia-induced pulmonary hypertension in newborn piglets

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Ananthakrishnan M, Barr FE, Summar ML, Smith HA, Kaplowitz M, Cunningham G, Magarik J, Zhang Y, Fike CD. L-Citrulline ameliorates chronic hypoxia-induced pulmonary hypertension in newborn piglets. *Am J Physiol Lung Cell Mol Physiol* 297: L506–L511, 2009. First published July 17, 2009; doi:10.1152/ajplung.00017.2009.— Newborn piglets develop pulmonary hypertension and have diminished pulmonary vascular nitric oxide (NO) production when exposed to chronic hypoxia. NO is produced by endothelial NO synthase (eNOS) in the pulmonary vascular endothelium using L-arginine as a substrate and producing L-citrulline as a byproduct. L-Citrulline is metabolized to L-arginine by two enzymes that are colocated with eNOS in pulmonary vascular endothelial cells. The purpose of this study was to determine whether oral supplementation with L-citrulline during exposure of newborn piglets to 10 days of chronic hypoxia would prevent the development of pulmonary hypertension and increase pulmonary NO production. A total of 17 hypoxic and 17 normoxic control piglets were studied. Six of the 17 hypoxic piglets were supplemented with oral L-citrulline starting on the first day of hypoxia. L-Citrulline supplementation was provided orally twice a day. After 10 days of hypoxia or normoxia, the animals were anesthetized, hemodynamic measurements were performed, and the lungs were perfused in situ. Pulmonary arterial pressure and pulmonary vascular resistance were significantly lower in hypoxic animals treated with L-citrulline compared with untreated hypoxic animals ($P < 0.001$). In vivo exhaled NO production ($P = 0.03$) and nitrite/nitrate accumulation in the perfusate of isolated lungs ($P = 0.04$) were significantly higher in L-citrulline-treated hypoxic animals compared with untreated hypoxic animals. L-Citrulline supplementation ameliorated the development of pulmonary hypertension and increased NO production in piglets exposed to chronic hypoxia. We speculate that L-citrulline may benefit neonates exposed to prolonged periods of hypoxia from cardiac or pulmonary causes.

nitric oxide synthase; nitric oxide; L-arginine recycling

INFANTS WITH CHRONIC LUNG DISEASE and cyanotic congenital heart disease frequently suffer from hypoxia. Because of its effects on both existing and developing pulmonary arteries, chronic hypoxia causes progressive changes in both the function and structure of the pulmonary circulation (28, 31). Ultimately, chronic hypoxia results in severe pulmonary hypertension culminating in right-sided heart failure and death. Currently, the therapy for pulmonary hypertension in infants suffering from chronic cardiopulmonary disorders associated with persistent or episodic hypoxia is largely limited to improving the underlying cardiopulmonary disorder and attempts to achieve adequate oxygenation (1, 2, 23, 31). The need for novel therapies to treat infants with chronic progressive neonatal pulmonary hypertension is well acknowledged (1–3, 14, 23).

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The piglet is an excellent species for the study of neonatal pulmonary hypertension since adaptation of the pulmonary circulation to extra-uterine life is similar in pigs and humans (14). Changes in pulmonary blood vessels found in piglets exposed to hypoxia approximate those found in human infants with pulmonary hypertension (15). We have previously shown that newborn piglets develop pulmonary hypertension when exposed to chronic hypoxia (9). Moreover, we have shown that the development of pulmonary hypertension in piglets exposed to 10 days of chronic hypoxia is associated with impaired production of the vasodilator nitric oxide (NO) (14).

NO is produced by endothelial NO synthase (eNOS) in the pulmonary vascular endothelium using L-arginine as a substrate and producing L-citrulline as a by-product. In turn, L-arginine can be synthesized from L-citrulline, providing a recycling pathway for the conversion of L-citrulline to NO via L-arginine (30). Plasmalemmal caveolae, the site of the L-citrulline-to-L-arginine recycling pathway, may be the principal source of L-arginine available to eNOS (12, 13, 30). Via this recycling pathway, the availability of L-citrulline may regulate NO production by eNOS in the pulmonary circulation.

The purpose of this study was to determine whether oral supplementation with L-citrulline during exposure of newborn piglets to 10 days of chronic hypoxia would prevent the development of pulmonary hypertension and the concomitant reduction in NO production.

METHODS

Animal care. All experimental protocols were performed in adherence with the National Institutes of Health guidelines for the use of experimental animals and approved by the Animal Care and Use Committee of Vanderbilt University Medical Center. The animal resource facility is fully accredited by the Association for Assessment and Accredidation of Laboratory Animal Care International. A total of 17 hypoxic and 17 normoxic control piglets were studied. Normoxic control animals were studied on the day of arrival from the farm at 12 days of age. The hypoxic pigs (2 days old) were placed in a normobaric hypoxic chamber for 10–11 days. Normobaric hypoxia was provided using compressed air and nitrogen to create inspired oxygen of 8–11% (P_{O_2} of 60–72 Torr) and CO_2 was maintained at 3–6 Torr by absorption with soda lime. The animals were monitored with daily weights and physical exam twice daily. They were fed ad libitum with sow milk replacer from a feeding device in the cage.

L-Citrulline supplementation. Six of the 17 hypoxic piglets were supplemented with oral L-citrulline starting on the first day of the hypoxic exposure. L-Citrulline supplementation was provided at a dose of 0.13 g/kg body wt twice a day using a syringe to deliver the dose orally. If it appeared to study personnel that the piglet had not ingested the majority of a dose, it was repeated. L-Citrulline was mixed using a preparation (Sigma Pharmaceuticals, 98% purity) at a concentration of 0.13 g/ml of distilled water. When completely dissolved, this solution was passed through a 0.20-μm filter.

In vivo hemodynamics. In vivo hemodynamics were measured in six of the normoxic control piglets and in all of the hypoxic piglets. For these measurements, the animals were weighed and then preanesthetized with Ketamine (15 mg/kg) and Acepromazine (2 mg/kg) intramuscularly. A tracheostomy, venous and arterial catheters, and thermistor were then placed as previously described using intravenous pentobarbital for sedation (10). Pulmonary artery pressure, left ventricular end diastolic pressure, and cardiac output were measured. Cardiac output was measured by a thermodilution technique (model 9520 thermodilution cardiac output computer, Edwards Laboratory, Irvine, CA) using a thermistor in the aortic arch and the left ventricle catheter as an injection port. Cardiac output was measured at end expiration as the mean of three injections of 3 ml of normal saline (0°C). Exhaled NO was measured as described below. During the in vivo measurements, animals were ventilated with room air using a piston-type ventilator at a tidal volume of 15–20 ml/kg, end-expiratory pressure of 2 Torr, and a respiratory rate of 15–20 breaths/min. Hemodynamic measurements were obtained in all hypoxic animals and six control animals. In our past experience as in this study, it is not always possible to obtain in vivo hemodynamic data on every animal for technical reasons. The most common difficulty encountered is the inability and length of time needed to place and advance a right heart catheter into the pulmonary artery to measure pulmonary artery pressure. Because of this difficulty, we did not attempt to obtain hemodynamic data in all control animals.

Exhaled NO measurement. For exhaled NO measurement in anesthetized animals, expiratory gas was sampled two to three times for 3-min periods each and passed through a chemiluminescence analyzer (model 270B NOA; Sievers, Boulder, CO) to measure NO concentration as previously described (11). Exhaled NO production (nmol/min) was calculated using minute ventilation and the measured exhaled NO concentration.

Isolated lung perfusions. All control and hypoxic animals used for hemodynamic measurements and an additional 11 control piglets were used in isolated lung perfusions. The lungs were isolated and perfused in situ with a Krebs Ringer bicarbonate (KRB) solution containing 5% dextran, molecular weight of 70,000, at 37°C and ventilated with a normoxic gas mixture (21% O_2 and 5% CO_2) as previously described (10). The lungs were perfused for 30–60 min until a stable pulmonary arterial pressure was achieved. Perfusate samples (1 ml) were then removed from the left atrial cannula every 10 min for a 60-min period. The perfusate samples were centrifuged, and the supernatant was stored at −80°C for future analysis of nitrite/nitrate (NOx^-) concentrations as described below. At the end of the perfusion, the volume of perfusate remaining in the circuit and reservoir was measured. In some cases, lung tissue was collected immediately following the perfusion, frozen with liquid nitrogen, and then stored at −80°C for later measurement of eNOS and nNOS content as described below. Isolated lung perfusions were attempted in all animals. It is our experience, as in this study, that it is not possible to successfully isolate and perfuse lungs in all animals for technical reasons.

NOx^- measurement. A chemiluminescence analysis described previously was used to determine perfusate NOx^- concentration (nmol/ml) at each collection time. (10, 34) Perfusate (20 μl) was injected into the reaction chamber of a chemiluminescence NO analyzer (model 170B NOA, Sievers). The reaction chamber contained vanadium (III) chloride in 1 M HCl heated to 90°C to reduce nitrite and nitrate to NO gas. The NO gas was carried into the analyzer using a constant flow of N_2 gas via a gas bubble trap containing 1 M NaOH to remove HCl vapor. A standard curve was generated by adding known amounts of $NaNO_3$ to distilled water and assaying as described for the perfusion samples.

The perfusate NOx^- concentration (nmol/ml) was calculated for each collection time by multiplying the perfusate concentration of NOx^- at that sample collection time by the volume of the system (perfusion circuit + reservoir) at the sample collection time plus the amount of NOx^- removed with all previous samples. The rate of NOx^- production was determined from the slope of a linear regression line fit to the amount of NOx^- in the perfusate vs. time for the first 60 min of the collection period.

Plasma amino acid measurements. On the day of hemodynamic measurements and/or lung perfusion study, for normoxic control and both L-citrulline-treated and -untreated chronic hypoxic animals, blood was drawn before the study was started and the plasma frozen at −80°C for later determination of amino acid levels. For the L-citrulline-treated hypoxic animals, a blood sample was obtained 12 h after the last dose of citrulline to measure the trough level of this amino acid. We wanted to verify that L-citrulline levels in treated animals were greater than those in untreated animals. Therefore, in some of the L-citrulline-treated animals ($n = 3$), after blood sampling for a trough level, a dose of L-citrulline was given via nasogastric tube. Following this dose, blood samples were drawn every 30 min for 90 min (the length of the in vivo studies). All samples were spun, and the plasma was collected and frozen at −80°C for amino acid analysis.

Concentrations of plasma citrulline and arginine were determined by amino-acid analysis on protein-free extracts. Amino acids were separated by cation-exchange chromatography using a Hitachi L8800 amino acid analyzer (Hitachi USA, San Jose, CA). Calibration of the analyzer was performed before piglet samples were tested.

Western blot of eNOS and nNOS in lung tissue. Using a standard immunoblot technique as previously described, we analyzed samples of whole lung homogenates from normoxic controls ($n = 3$) and untreated hypoxic ($n = 3$) and L-citrulline-treated hypoxic ($n = 3$) animals for eNOS and nNOS. We used 10 and 30 mg of total protein for eNOS and nNOS, respectively, a dilution of primary eNOS or nNOS antibody of 1:500 (BD transduction), and a dilution of secondary anti-mouse antibody conjugated to horseradish peroxidase of 1:5,000 (11).

Calculations and statistics. Pulmonary vascular resistance was calculated from the in vivo hemodynamic measurements: (pulmonary arterial pressure − left ventricular end diastolic pressure) ÷ (cardiac output/body wt).

Data are presented as means ± SD. The one-way ANOVA with Fisher's protected least significant difference (PLSD) post hoc comparison test was used to compare data between normoxic control and untreated hypoxic and L-citrulline-treated hypoxic animals. A P value of <0.05 was considered significant (21).

RESULTS

In vivo hemodynamic measurements. Both L-citrulline-treated and -untreated chronic hypoxic animals had lower cardiac output and weights and higher left ventricular end-diastolic pressure measurements on the day of study at 12–13 days of age than comparable age normoxic control piglets (Table 1). We have previously shown that piglets grown under hypoxic conditions have less weight gain than those grown under normoxic conditions (9). Measurements of aortic pressure and arterial P_{O_2} (Pa_{O_2}) were similar (Pa_{O_2} was 74 ± 13 Torr in normoxic control piglets, 74 ± 16 Torr in untreated hypoxic piglets, and 78 ± 16 Torr in L-citrulline-treated hypoxic piglets) among groups. Values for arterial P_{CO_2} (Pa_{CO_2}) were significantly lower ($P = 0.04$) in the L-citrulline-treated hypoxic animals (30 ± 3 Torr) compared with both normoxic controls (39 ± 6 Torr) and untreated hypoxic (41 ± 12 Torr) animals. However, since the values of pH did not differ significantly between any of the groups of animals (Table 1), these differences in Pa_{CO_2} are unlikely to have had any physiological impact on the hemodynamic measurements.

Notably, as shown in Fig. 1*A*, L-citrulline-treated hypoxic animals had significantly lower pulmonary artery pressures

Table 1. *Data for normoxic control, chronically hypoxic, and L-citrulline-treated chronically hypoxic piglets*

Treatment Group	Weight at 12 Days of Age, kg	Aortic Pressure, cmH_2O	LVEDP, cmH_2O	Cardiac Output, $ml \cdot min^{-1} \cdot kg^{-1}$	Arterial pH
Controls (n = 6)	3.94±0.7	91±9	5.2±1.5	414±105	7.38±0.12
Chronic hypoxic (n = 11)	2.76±0.5*	100±12	7.4±1.7*	244±00*	7.38±0.04
Citrulline hypoxic (n = 6)	2.6±0.23*	97±15	7.2±1.1*	270±71*	7.36±0.05

Values are means ± SD. LVEDP, left ventricular end-diastolic pressure. *Significnat difference vs. normoxic controls ($P < 0.05$; ANOVA with post hoc comparison test).

than untreated hypoxic animals. In addition, as shown in Fig. 1*B*, calculated pulmonary vascular resistance in those hypoxic animals treated with L-citrulline were significantly lower than those of untreated hypoxic animals. Furthermore, pulmonary vascular resistances were similar in L-citrulline-treated hypoxic animals and normoxic controls.

Exhaled NO output and perfusate NOx^-. As shown in Fig. 2*A*, exhaled NO output in normoxic controls and L-citrulline-treated hypoxic animals were higher than exhaled NO output in untreated hypoxic animals. However, exhaled NO output did not differ between normoxic control and L-citrulline-treated hypoxic animals.

As shown in Fig. 2*B*, lungs from both the normoxic control and L-citrulline-treated hypoxic animals had significantly higher NOx^- accumulation rates than lungs from untreated hypoxic animals. Furthermore, there was no difference in the rate of NOx^- accumulation between lungs from L-citrulline-treated hypoxic animals and normoxic controls.

Plasma amino acids. As shown in Table 2, although not reaching statistical significance, plasma L-citrulline levels in untreated chronic hypoxic piglets were less than trough L-citrulline levels in treated hypoxic piglets. Moreover, when drawn 90 min after a dose, levels of L-citrulline in treated hypoxic animals were almost twice that of the untreated

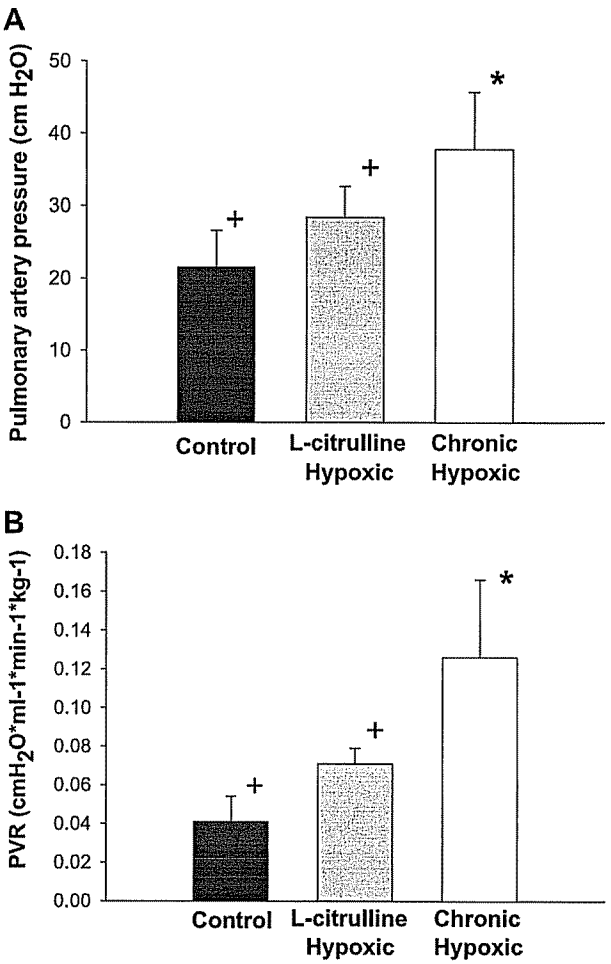

Fig. 1. *A*: mean pulmonary arterial pressure measurements in normoxic control (n = 6), chronically hypoxic (n = 11), and L-citrulline-treated chronically hypoxic (n = 6) piglets. *B*: calculated pulmonary vascular resistance in normoxic control (n = 6), chronically hypoxic (n = 11), and L-citrulline-treated chronically hypoxic (n = 6) piglets. Values are means ± SD. Significantly different from normoxic control (*) and chronically hypoxic (+) ($P < 0.05$; ANOVA with post hoc comparison test).

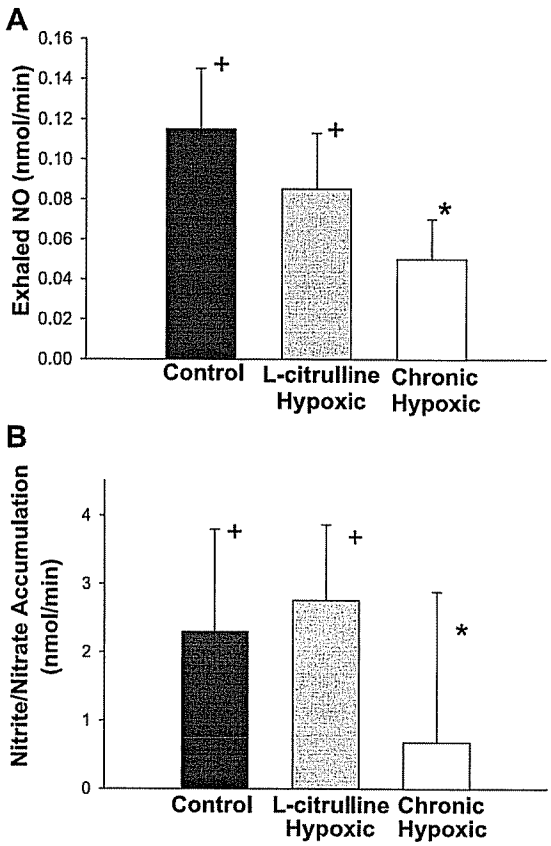

Fig. 2. *A*: exhaled nitric oxide in normoxic control (n = 6), chronically hypoxic (n = 11), and L-citrulline-treated chronically hypoxic (n = 5) piglets. *B*: nitrite/nitrate accumulation in lung perfusate in normoxic control (n = 17), chronically hypoxic (n = 9), and L-citrulline-treated chronically hypoxic (n = 5) piglets. Values are means ± SD. Significantly different from *normoxic control (*) and chronically hypoxic (+) ($P < 0.05$; ANOVA with post hoc comparison test).

Table 2. *Plasma amino acid levels for normoxic control, chronically hypoxic, and L-citrulline-treated chronically hypoxic piglets*

Treatment Group	Citrulline, μM	Arginine, μM
Normoxic controls (n =10)	71±20	112±49
Chronic Hypoxic (n =8)	111±67	51±31*
L-Citrulline-treated hypoxic (90 min; n =3)	219±63*†	43±8*
L-Citrulline-treated hypoxic (trough; n = 6)	161±13*	39±24*

Values are means ± SD. Trough, plasma level ~12 h after L-citrulline dose; 90 min, plasma level 90 min after administration of L-citrulline dose. *Significant difference vs. normoxic controls ($P < 0.05$; ANOVA with post hoc comparison test). †Significant difference vs. untreated chronic hypoxics ($P < 0.05$; ANOVA with post hoc comparison test).

chronic hypoxic animals. Levels of L-citrulline obtained at 30 (135 ± 60 μM) and 60 (156 ± 9 μM) min after a dose did not differ significantly from the 90-min value. Regardless of the time the sample was drawn, plasma arginine levels were not higher in L-citrulline-treated chronic hypoxic animals compared with untreated hypoxic animals.

Western blot for lung eNOS and nNOS protein. As shown in Fig. 3 and consistent with our previous studies (11), the amount of eNOS protein present in the lung tissue of normoxic control animals was significantly higher than that present in the lungs of untreated hypoxic animals. Furthermore, the amount of eNOS protein present in the lung tissue of L-citrulline-treated hypoxic piglets was not significantly different from that in the untreated hypoxic animals and was significantly lower than eNOS protein levels in normoxic control animals. As shown in Fig. 4, there was no difference in nNOS protein levels among the three groups.

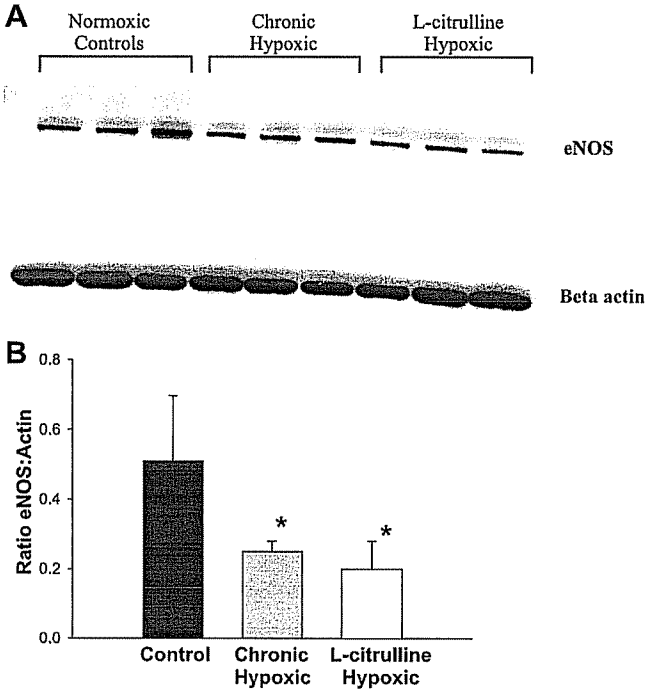

Fig. 3. *A*: immunoblot for eNOS protein reprobed for beta actin for lung tissue from normoxic controls (n = 3), chronic hypoxic (n = 3), and L-citrulline-treated chronic hypoxic (n = 3) piglets. *B*: densitometry of eNOS normalized to beta actin for lung tissue from normoxic controls (n = 3), chronic hypoxic (n = 3), and L-citrulline-treated chronic hypoxic (n = 3) piglets Values are means ± SD. *Significantly different from normoxic control ($P < 0.05$; ANOVA with post hoc comparison test).

DISCUSSION

In this study, we found that L-citrulline supplementation ameliorates the development of pulmonary hypertension in newborn piglets exposed to 10 days of chronic hypoxia. To our knowledge, this is the first study showing the effectiveness of L-citrulline in preventing the development of pulmonary hypertension in either newborn or more mature animal models of this disease.

Other important findings in this study are that both exhaled NO production and pulmonary vascular NOx^- accumulation rates are greater in L-citrulline-treated hypoxic piglets than in untreated hypoxic piglets. Thus our findings clearly show that L-citrulline supplementation significantly increased pulmonary NO production. In addition, our finding that the amounts of eNOS and nNOS protein are unchanged in the L-citrulline-treated hypoxic animals suggests that the mechanism for this increase in pulmonary NO production is not an increase in NOS expression.

Based on the current literature (13, 17 ,30 ,32), the mechanism by which L-citrulline mediates an increase in NO production could be by improving NOS function. One possible mechanism for improving NOS function is by increasing the amount of L-arginine available as a substrate for eNOS. Assessment of arginine availability for NO synthesis has been a challenge that has been addressed by many investigators. Plasma levels of arginine in the L-citrulline-treated animals in this study were not significantly increased compared with untreated hypoxic animals. However, this finding was not surprising since total cellular levels of L-arginine have not been

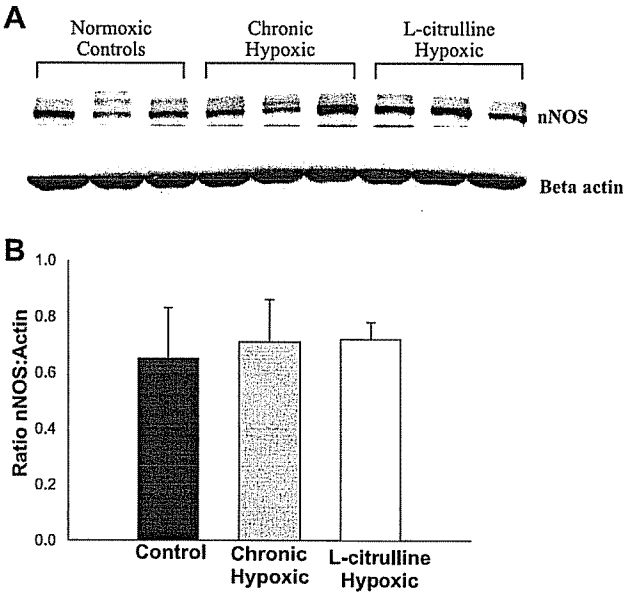

Fig. 4. *A*: immunoblot for nNOS protein reprobed for beta actin for lung tissue from normoxic controls (n = 3), chronic hypoxic (n = 3), and L-citrulline-treated chronic hypoxic (n = 3) piglets. *B*: densitometry of nNOS normalized to beta actin for lung tissue from normoxic controls (n = 3), chronic hypoxic (n = 3), and L-citrulline-treated chronic hypoxic (n = 3) piglets. Values are means ± SD.

found to accurately reflect subcellular levels of L-arginine available for NO synthesis. Su and Block (32) attempted to show that decreased NO production in pulmonary endothelial cells exposed to hypoxia was due to a decrease in cellular L-arginine content. They found that, rather than being decreased, cellular L-arginine content was actually increased by degradation of cellular proteins in response to hypoxia and hypothesized that this increased supply of L-arginine was unavailable to eNOS (32). Solomonson et al. in 2003 showed that providing L-arginine to endothelial cells increased NO production only slightly compared with the more dramatic increase in endothelial NO production found with L-citrulline supplementation (30). In addition, L-citrulline supplementation increased total cellular arginine only slightly compared with the significant increase in total cellular arginine after L-arginine supplementation. Thus, similar to Su and Block, these authors concluded that there was no correlation between total cellular arginine and endothelial NO production (30). Based on findings from these and other studies (13, 17), eNOS function seems to be dependent on a pool of arginine that is isolated from the bulk of intracellular arginine and is maintained through an efficient arginine regeneration enzymatic process in close proximity to eNOS.

This discordance between intracellular arginine and NO production, termed the "arginine paradox," explains the increase in NO production in the face of unchanged plasma arginine levels seen with L-citrulline supplementation in this study. L-Citrulline is a urea cycle intermediate metabolized to arginine by a recycling pathway consisting of two enzymes, argininosuccinate synthase (AS) and argininosuccinate lyase (AL). These two enzymes, AS and AL, have been found colocated with eNOS in pulmonary endothelial cells (7). It is thought that together these enzymes produce a separate subcellular pool of arginine used exclusively for NO synthesis. Tissue and plasma arginine levels cannot accurately measure this subcellular pool.

L-Citrulline may also have improved NO production and eNOS function by additional mechanisms. Recently, it has been suggested that, in the setting of ischemia and reperfusion injury, the enzyme eNOS (a dimer) uncouples and produces superoxide instead of NO (7). There is evidence that this uncoupling of eNOS occurs in the presence of low levels of arginine or BH4, a necessary cofactor for the production of NO (35). Hence, another potential action of L-citrulline in this study is the prevention of the uncoupling of eNOS by maintaining adequate levels of its substrate arginine. We have yet to explore this possibility.

L-Citrulline has been used in several patient populations with some success. In addition to those patients with urea cycle defects, patients with sickle cell disease receiving citrulline have shown improved disease symptoms (36). In children undergoing cardiopulmonary bypass at risk for development of postoperative pulmonary hypertension, Smith et al. recently showed that oral supplementation with L-citrulline increased both plasma citrulline and arginine levels (29). Moreover, postoperative pulmonary hypertension did not develop in those children who had plasma citrulline levels greater than 37 μM/l. Furthermore, intravenous L-citrulline has been shown to be safe and well tolerated in this same patient population of children undergoing bypass by Barr et al. (4).

Notably, L-citrulline therapy has been used in animal models of vascular diseases other than our model of chronic hypoxia-induced pulmonary hypertension. In rabbits fed a high-cholesterol diet, L-citrulline supplementation causes regression of atheromatous lesions (16). In spontaneously hypertensive rats, maternal supplementation with L-citrulline increased renal NO production and ameliorated hypertension in offspring (18). Therefore, it would seem that L-citrulline may be useful for improving NO dysfunction in conditions other than hypoxia-induced pulmonary hypertension.

Although L-citrulline has not been widely studied as a therapy for pulmonary hypertension, L-arginine supplementation has been used frequently with mixed results. For example, treatment with L-arginine has been shown to prevent the development of pulmonary hypertension in two adult rat models of pulmonary hypertension (22, 25). Furthermore, administration of L-arginine was shown to reverse evidence of postoperative pulmonary vascular endothelial dysfunction in children who had undergone cardiopulmonary bypass and to restore impaired pulmonary vasorelaxation in adults with pulmonary hypertension (6, 8, 20, 24, 27). Although these studies provide evidence that L-arginine may help prevent the development of pulmonary hypertension and may be helpful once pulmonary hypertension has developed, serious adverse effects of L-arginine treatment have been suggested, and variable results from L-arginine treatment have been reported (5, 26). Because arginine is involved in other processes in the body and is quickly metabolized by arginases in many cellular compartments, supplementation often requires high doses, i.e., 9 g/day, in adults (26). These massive doses are sometimes poorly tolerated, and patient compliance can be difficult to maintain (33).

There are several limitations of this study that merit comment. First, we have been unable to detect iNOS protein in lung tissue from newborn piglets using those antibodies currently commercially available. Thus, although we have shown that eNOS and nNOS protein levels in lung tissue are unchanged with L-citrulline therapy, we cannot rule out the possibility that an increase in iNOS protein contributes to the increase in NO production and decrease in pulmonary vascular resistance in L-citrulline-treated hypoxic piglets. In addition, eNOS has been shown to be present in respiratory epithelium as well as pulmonary vascular endothelium (34). Therefore, Western blots of whole lung homogenates cannot establish the precise anatomical site of any change in lung eNOS expression.

Another study limitation is that we did not measure AS and AL amounts or activities. It is possible that changes in the amount or activity of these enzymes that are colocated with eNOS could contribute to alterations in NO production. Yet another limitation is that our study findings do not address the possibility that L-citrulline therapy may have effects in normoxic animals. Also, because isolated lung perfusion requires disruption of the right ventricle morphology and can cause edema and distortion of the pulmonary architecture, we were unable to assess the effect of L-citrulline therapy on either right ventricular hypertrophy or pulmonary vascular remodeling. We were unable to assess the changes in pulmonary vasoreactivity since the agonists used to determine reactivity can potentially alter lung NO production. In addition, vessels harvested from isolated perfused lung preparations are no longer viable for use in pressurized, cannulated artery studies.

Further studies are required to more extensively evaluate the mechanisms underlying the effect of L-citrulline therapy on NOS function, potential changes in vasoreactivity, and the development of pulmonary hypertension.

In summary, our findings show that L-citrulline ameliorates chronic hypoxia-induced pulmonary hypertension in newborn piglets. We also provide evidence that the effectiveness of citrulline is due to increased NO production, which is likely due at least in part to an increase in NOS function since neither eNOS nor nNOS protein levels are changed. It is possible that L-citrulline may be a useful therapy in neonates at risk of developing pulmonary hypertension due to conditions associated with impaired NO function, including chronic or intermittent unresolved hypoxia.

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